JOURNAL OF VIROLOGY, July 1982, p. 284–293
0022-538X/82/070284-10$02.00/0

Vol. 43, No. 1

Infectious Cell Entry Mechanism of Influenza Virus

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Received 24 November 1981/Accepted 31 March 1982

Interaction between influenza virus WSN strain and MDCK cells was studied by using spin-labeled phospholipids and electron microscopy. Envelope fusion was negligibly small at neutral pH but greatly activated in acidic media in a narrow pH range around 5.0. The half-time was less than 1 min at 37°C at pH 5.0. Virus binding was almost independent of the pH. Endocytosis occurred with a half-time of about 7 min at 37°C at neutral pH, and about 50% of the initially bound virus was internalized after 1 h. Electron micrographs showed binding of virus particles in coated pits in the microvillous surface of plasma membrane and endocytosis into coated vesicles. Chloroquine inhibited virus replication. The inhibition occurred when the drug was added not later than 10 min after inoculation. Chloroquine caused an increase in the lysosomal pH 4.9 to 6.1. The drug did not affect virus binding, endocytosis, or envelope fusion at pH 5.0. Electron micrographs showed many virus particles remaining trapped inside vacuoles even after 30 min at 37°C in the presence of drug, in contrast to only a few particles after 10 min in vacuoles and secondary lysosomes in its absence. Virus replication in an artificial condition, i.e., brief exposure of the inoculum to acidic medium followed by incubation in neutral pH in the presence of chloroquine, was also observed. These results are discussed to provide a strong support for the infection mechanism of influenza virus proposed previously: virus uptake by endocytosis, fusion of the endocytosed vesicles with lysosome, and fusion of the virus envelope with the surrounding vesicle membrane in the secondary lysosome because of the low pH. This allows the viral genome to enter the target cell cytoplasm.

Enveloped viruses must uncoat to transfer their genomes into the target cell cytoplasm. Hemagglutinating virus of Japan (a synonym of Sendai virus) and other paramyxoviruses do this by envelope fusion with the target cell plasma membrane (envelope fusion from outside) (30). The uncoating mechanism of influenza virus (orthomyxovirus), however, has not yet been made clear. Most of the morphological studies have shown uptake of the virus by endocytosis (2, 4, 29), but one study suggested envelope fusion (26). Fusion of liposomes containing the viral glycoproteins with cells has also been reported (11). Moreover, the uncoating mechanism after endocytosis has not been clarified.

We have found that envelope fusion of influenza virus with erythrocytes is negligibly small at neutral pH but vigorously activated by lowering pH to 5.2 (24). Hemolysis and cell fusion were also activated in the same pH range. The cleaved form of viral hemagglutinin, but not the uncleaved precursor form, is required for the acid activation (22). Based on these findings, we proposed the following uncoating mechanism (22, 24). After cell entry by endocytosis, the endocytosed vesicles fuse with lysosomes. The virus envelope then fuses rapidly with the surrounding vesicle membrane because of low pH inside the lysosome. The viral RNA is transferred into cytoplasm by this fusion event (envelope fusion from inside). This mechanism can reasonably explain the uncoating because the intralysosomal pH was reported as 4.7 to 4.8 for macrophage (27). This cell entry mechanism is essentially the same as that proposed for Semliki Forest virus, a togavirus, by Helenius et al. (9, 35). Huang et al. (10) also observed extensive hemolysis and cell fusions in acid by influenza virus and requirement of the cleaved form of hemagglutinin for the activity. Lenard and Miller (19) also showed influenza virus-induced hemolysis in acid. White et al. (36) observed fusion of cultured cells in acid by influenza virus as well as Semliki Forest virus and vesicular stomatitis virus.

The present study was undertaken to confirm the proposed infectious cell entry mechanism by using influenza virus WSN strain and Madin-Darby canine kidney (MDCK) cells. We studied endocytosis, envelope fusion and cell fusion in

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acid, replication of virus, and effect of lysosomotropic weak bases by using spin-labeled phospholipids and electron microscopy. The results provide a strong support for the mechanism.

MATERIALS AND METHODS

Cells. MDCK cells and baby hamster kidney (BHK) cells were grown at 37°C under 5% CO_2 in 60-mm plastic petri dishes (Nunc) in Eagle minimum essential medium (MEM) supplemented with 10% newborn calf serum, penicillin (20 U/ml), streptomycin (0.1 mg/ml), and kanamycin (60 μg/ml). Cells were released from the plastic dishes with 0.1% trypsin (Difco) in 1 mM EDTA in phosphate-buffered saline (PBS) for about 10 min at 37°C.

Virus. Influenza virus WSN (H1N1) strain was grown in the allantoic cavity of 10-day-old embryonated eggs for 48 h at 36°C and purified by centrifugation as described previously (20). Virus concentration was expressed as hemagglutinating units (HAU) or PFU. Hemagglutination titration was done by Salk's pattern method (32). Plaque assay was performed on MDCK monolayers grown in plastic dishes (6). Virus samples were serially diluted in PBS containing 1.0% bovine serum albumin, and 0.2-ml fractions were adsorbed onto the monolayers for 30 min at 37°C. The cell cultures received 5 ml of agar overlay medium consisting of reinforced Eagle medium (1) supplemented with bovine serum albumin (0.2%), DEAE-dextran (0.1 mg/ml), trypsin (10 μg/ml), glucose (1 mg/ml), and agar (Difco; 1.0%). After 3 days of incubation at 37°C under CO_2, plaques were detected by staining with crystal violet (1 mg/ml) in 20% ethanol. The ratio of PFU to HAU was 3.9×10^5 for the infected allantoic fluid. If we use 1.6×10^7 for average number of virus particles per HAU (3), 1 PFU is equivalent to 50 to 100 virus particles.

Spin-labeling of virus was carried out as described previously (20, 21). Briefly, PC* or Tempo-PC (see Fig. 1) was suspended in Tricine-buffered saline at 1 mM, sonicated for 5 min on ice, and centrifuged for 1 h at 100,000 × g. The supernatant (1 ml) was added to virus (about 50,000 HAU) and incubated for 5 h (PC*) or 2 h (Tempo-PC) at 37°C. Spin-labeled virus was washed with 1% bovine serum albumin in PBS and then with PBS. Hemolysis activity of the labeled viruses was nearly the same as that of unlabeled viruses. Tempo-PC was synthesized by condensation of tempocholine with phosphatidic acid derived from egg yolk lecithin (17).

Assay of hemolysis and cell fusion. Virus-induced hemolysis and cell fusion were assayed at varying pH values. Human erythrocytes or BHK cells released from the monolayer culture were suspended in balanced salt solution (BSS; 140 mM NaCl, 54 mM KCl, 2 mM $CaCl_2$, 0.44 mM KH_2PO_4, 0.34 mM Na_2HPO_4) at a concentration of 2.5% (vol/vol) or 2×10^7 cells per ml, respectively. The cell suspension was mixed with virus (final concentrations, 3,000 HAU/ml for hemolysis and 6,000 HAU/ml for cell fusion), kept on ice for 1 h, and centrifuged for 5 min at 2,000 rpm at 4°C. The pellet was suspended in BSS buffered with 10 mM MES [2-(morpholino)ethane sulfonic acid; Dotite] for pH values of 4.75 to 6.0 or with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Nakarai) for pH values of 6.5 to 7.5, and incubated for 30 min at 37°C with shaking. Hemolysis was determined spectrophotometrically at 540 nm. Cell fusion was observed under an optical microscope, and the fusion index, defined as [(total cell number before incubation/total cell number after incubation) − 1], was calculated (28).

Assay of envelope fusion. Envelope fusion was assayed by measuring increase in the central peak height of electron spin resonance (ESR) spectrum when PC*-labeled virus was incubated with cells as described previously (20, 21, 23, 25). Typically, 3,000 HAU of PC*-labeled virus per ml was mixed with MDCK cell suspension (8×10^6 cells per ml) in PBS, kept on ice for 20 min for adsorption, and centrifuged for 2 min at 2,000 rpm. The pellet was washed with BSS at various pH values, and the ESR spectrum was measured repeatedly at 37°C. When envelope fusion occurred,

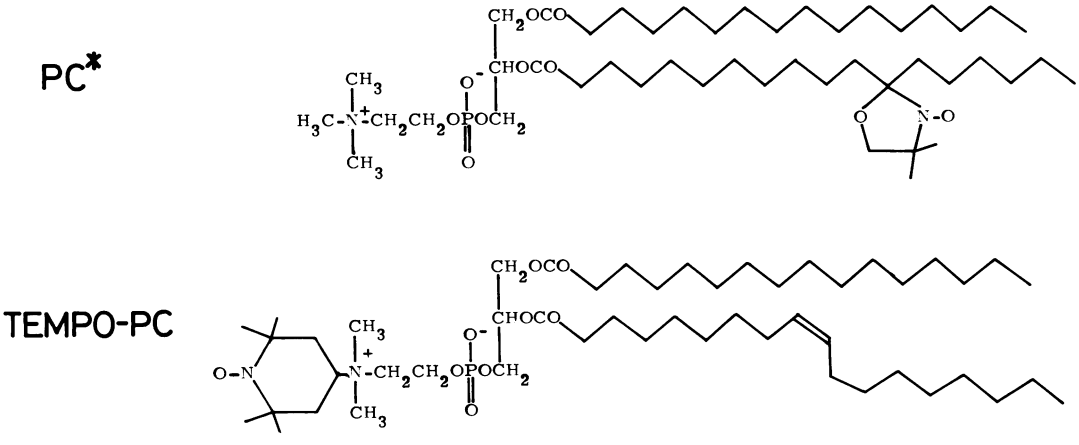

FIG. 1. Spin-labeled phospholipid used for the assay of phospholipid transfer (PC*) and virus remaining bound on the cell surface (Tempo-PC).

PC* preincorporated into virus envelope rapidly diffused away from the fused site and intermixed well with the target cell membrane lipid. Since the virus envelope contained a high concentration of PC* (12 to 15% of viral lipid), the intermixing caused dilution of PC*, which weakened the spin-spin exchange interaction and led to increase in the ESR peak height. The peak height increase can be written by $P_t/P_0 = f - (f - 1)e^{-kt}$, and the initial slope is indicated by $(f - 1)k$, where P_t and P_0 represent the central peak height at times t and 0, respectively, and k is the rate constant for envelope fusion. Further, f is the peak height increase factor, i.e., $f = P_c/P_v$, where P_c and P_v are the central peak heights for PC* in the virus envelope and cell membrane, respectively; this was determined to be 7 to 8 in our experimental conditions. The peak height increase was approximately proportional to the fraction of virus that fused with cell membrane. This was shown by adding model spectra for virus and cells at various ratios and plotting the central peak height against fraction of the virus spectrum. An approximately linear relationship was obtained between them.

Assay of virus remaining on the cell surface. Tempo-PC incorporated into virus envelope was readily reduced by ascorbate because the spin label nitroxide moiety was exposed to the aqueous phase. The ESR signal disappeared very rapidly (within a minute) on addition of 10 mM ascorbate. When the virus was washed and added to 10 mM ferricyanide, the reduced nitroxide (the hydroxylamine) was reoxidized immediately to restore the ESR signal. We assayed virus particles remaining bound on the cell surface on this principle.

Tempo-PC-labeled virus was pretreated at 4°C with 10 mM ascorbate (Wako Chemicals) in Hanks buffered with 20 mM HEPES at pH 7.5 and washed. The virus (final concentration, 3,000 HAU/ml) was allowed to adsorb to MDCK cells suspended in PBS (final concentration 5×10^6 cells per ml) for 1 h at 4°C and centrifuged. The pellet was suspended in MEM-HEPES at pH 7.4, incubated at 37°C for varying periods with shaking, and pelleted by centrifugation for 5 min at 2,000 rpm at 4°C. The pellet was added to 5 μl of 10 mM ferricyanide in Hanks buffered with 20 mM HEPES at pH 7.5, and the ESR spectrum was measured at room temperature (22 to 23°C) within a few minutes. The restored ESR signal was defined as the virus particles remaining bound on the cell surface.

When the ascorbate-treated Tempo-PC-virus was incubated with cells in the same way as above and the pellet was resuspended in the same buffer but lacking ferricyanide, no ESR signal was detected. This indicates that the ascorbate-reduced nitroxides were not reoxidized by the cellular materials and verifies that the restored signal is due to oxidation by ferricyanide. To confirm that the restored signal comes from viruses bound on the cell surface but not from those endocytosed, the inoculum was incubated in MEM-HEPES at pH 7.4 for 30 min at 37°C and then for a further 30 min in the same medium with or without neuraminidase (5 mg/ml, Sigma type V). The ESR peak height from the neuraminidase-treated cells was about 50% of that from the nontreated cells. A separate experiment showed that the neuraminidase treatment removed approximately 50% of the bound virus from the cell surface.

pH dependence of the virus binding was determined by the same technique. The ascorbate-treated Tempo-PC-virus was allowed to adsorb to MDCK cell suspension for 1 h on ice in BSS at various pH values. After washing with PBS, the pellet was resuspended in 5 μl of 10 mM ferricyanide in Hanks-HEPES at pH 7.5, and the ESR spectrum was measured.

For comparison, the virus binding was also assayed using ^{125}I-labeled virus by the method of Fraker and Speck (5). The radiolabeled virus (3,200 HAU/ml) was mixed with cell suspension (5×10^5 cells per ml) in MEM-HEPES at various pH values and incubated for 1 h at 4°C. The result gave an order of magnitude of binding and a pH dependence similar to those obtained by the spin-label method. Attempts to assay endocytosis by radiolabeled viruses were not successful for the present system because neuraminidase and protease treatments of the virus-cell suspensions only partially released the surface-bound viruses.

Effect of drugs on virus replication. MDCK cell monolayers in plastic petri dishes (4×10^6 to 5×10^6 cells) were preincubated in MEM-HEPES at neutral pH, containing drugs, for 30 min at 37°C. Virus was added to the monolayers at a multiplicity of 10 PFU/cell and kept for 1 h at 4°C. The inoculum was washed, fresh MEM containing or not containing drugs was added, and cells were incubated further at 37°C. When the virus yield was assayed by plaque formation, trypsin (5 μg/ml) was added to the medium for final incubation to activate the virus infectivity (14, 18).

A variety of lysosomotropic weak bases (chloroquine [Sigma], NH_4Cl, methylamine, lidocaine [Sigma], neutral red [Nakarai]), cytoskeletal drugs (colchicine [Merck] and cytochalasin B [Sigma]), and calmodulin inhibitors [trifluoroperazine (Yoshitomi Seiyaku) and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W-7, a gift of H. Hidaka)] were used. These drugs were dissolved in MEM-HEPES, at pH 7.8 for lysosomotropic drugs and at pH 7.5 for other drugs. Sulfatides (anionic trehalose glycolipids) were extracted by the method of Goren (7) from *Mycobacterium tuberculosis* provided by M. Kino. The sulfatides were suspended in MEM at 0.5 mg/ml and sonicated for 5 min at 4°C. Intracellular chloroquine concentration was determined by fluorometry at 370 nm with excitation at 340 nm after cells were solubilized in 1% deoxycholate at pH 11.3 (31). Intralysosomal pH was determined from the ratio of fluorescence intensity at 495 nm to that at 553 nm after incubation of cells with fluorescein isothiocyanate-conjugated dextran (2 mg/ml, Sigma FD-40) for 48 h at 37°C and washing according to Ohkuma and Poole (27).

Virus replication by brief exposure to acidic media. MDCK cell monolayers were preincubated for 30 min at 37°C in MEM-HEPES (pH 7.8) containing 0.1 mM chloroquine. Virus was allowed to adsorb to the monolayers at 4°C for 1 h at varying multiplicities from 10 to 100 PFU/cell and then exposed for 2 min at 37°C to acidic medium (BSS-MES or BSS-HEPES) containing 0.1 mM chloroquine. The cells were washed with MEM-HEPES (pH 7.8) containing chloroquine and incubated for 3 h at 37°C. The medium was then replaced with fresh MEM not containing chloroquine, and the cells were incubated for a further 6 h at 37°C.

Electron microscopy. MDCK cell monolayers grown on cover slips were inoculated with virus at 10 or 100 PFU/cell and incubated for various periods at 37°C in MEM-HEPES at neutral pH and with or without 0.1

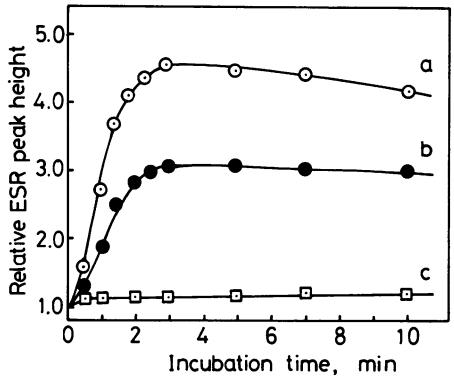

FIG. 2. Time course of ESR peak height increase at three different pH values: (a) 5.0, (b) 5.5, and (c) 7.0. PC*-labeled influenza virus (3,000 HAU/ml) was adsorbed to MDCK cells (8×10^6 cells per ml) at 4°C and then resuspended in BSS at the indicated pH values. The ESR spectrum was measured at 37°C repeatedly, and the central peak height at time t, divided by that at time 0, is plotted.

mM chloroquine. The cells were fixed with 2.5% glutaraldehyde in 100 mM sodium cacodylate for 1 h, postfixed in 1% osmic acid for 1 h, and dehydrated in a graded series of ethanol. Cells embedded in Epon 812 were removed from the cover slip by dipping into liquid nitrogen. Ultrathin sections were cut parallel to the cover slip plane on a Porter-Blum MT-2B ultramicrotome and examined in a Hitachi HU-12A electron microscope after counterstaining with uranyl acetate and lead acetate.

RESULTS

Activation of envelope fusion and cell fusion in acid. Figure 2 shows the time course of the ESR peak height increase at various pH values when the PC*-labeled virus was incubated with MDCK cells at 37°C. In acidic media, the peak height increased rapidly and markedly in contrast to that at neutral pH. The increase is mainly due to intermixing of spin-labeled phospholipid with the target cell membrane lipids as a result of envelope fusion. The initial slope is proportional to the envelope fusion rate constant $[(f - 1)k$; see Materials and Methods]. Its pH dependence is shown in Fig. 3B. It is seen that the marked activation of envelope fusion occurred in a relatively narrow pH range around 5.0. The virus-induced hemolysis and cell fusion were also markedly activated in acidic media (Fig. 3C). The pH dependence was quite similar to that of envelope fusion, as expected. On the other hand, the virus binding to the cell surface was not much affected by the medium pH (Fig. 3A).

Chloroquine did not affect the envelope fusion in acid; the time course of ESR peak height increase at pH 5.0 in the presence of 0.1 mM chloroquine was superposable to that in its absence. The virus binding was not affected by chloroquine either.

Endocytosis. Reactions between the virus and MDCK cells were followed by electron microscopy and spin-labeling. When the virus was added to the cell monolayer and kept on ice, most of the virus particles adsorbed onto the microvillous surface of the plasma membrane (Fig. 4a). When warmed to 37°C, the cells rapidly took up the virus by endocytosis. After 5 min, the virus was engulfed in coated pits, mostly at

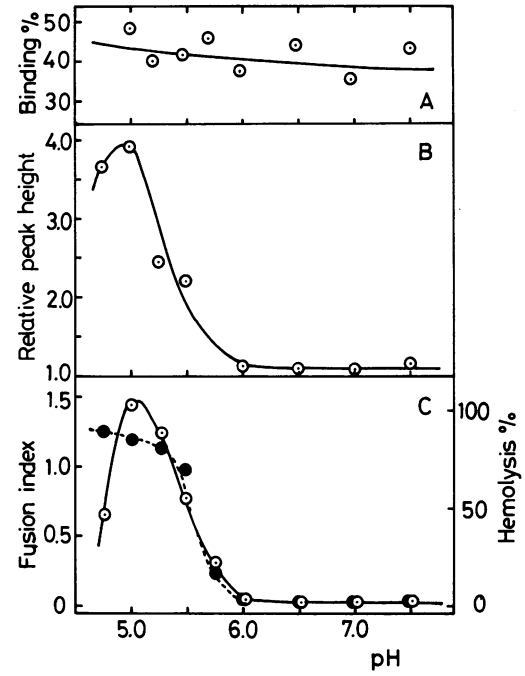

FIG. 3. pH dependence of (A) virus binding, (B) envelope fusion, and (C) virus-induced cell fusion (○) and hemolysis (●). (A) Tempo-PC-labeled virus (3,000 HAU/ml) was added to MDCK cell suspension (5×10^6 cells per ml) at various pH values, kept for 1 h at 4°C, and washed with PBS at 4°C. The pellet was resuspended in ferricyanide, and the ESR spectrum was measured at room temperature within minutes. The percentage of binding was calculated by comparison of the ESR signal height with that of a known concentration of the virus suspension. (B) PC*-labeled virus was mixed with MDCK cell suspension at various pH values and the ESR spectrum was measured at 37°C repeatedly (see Fig. 2). The ordinate gives the peak height at 90 s, which is approximately proportional to the envelope fusion rate constant. (C) Virus was mixed with human erythrocytes or BHK cells at 4°C in neutral pH medium and allowed to adsorb for 1 h. The pellet was resuspended in BSS buffered at various pH values and incubated for 30 min at 37°C. Hemolysis and cell fusion were assayed as described in the text.

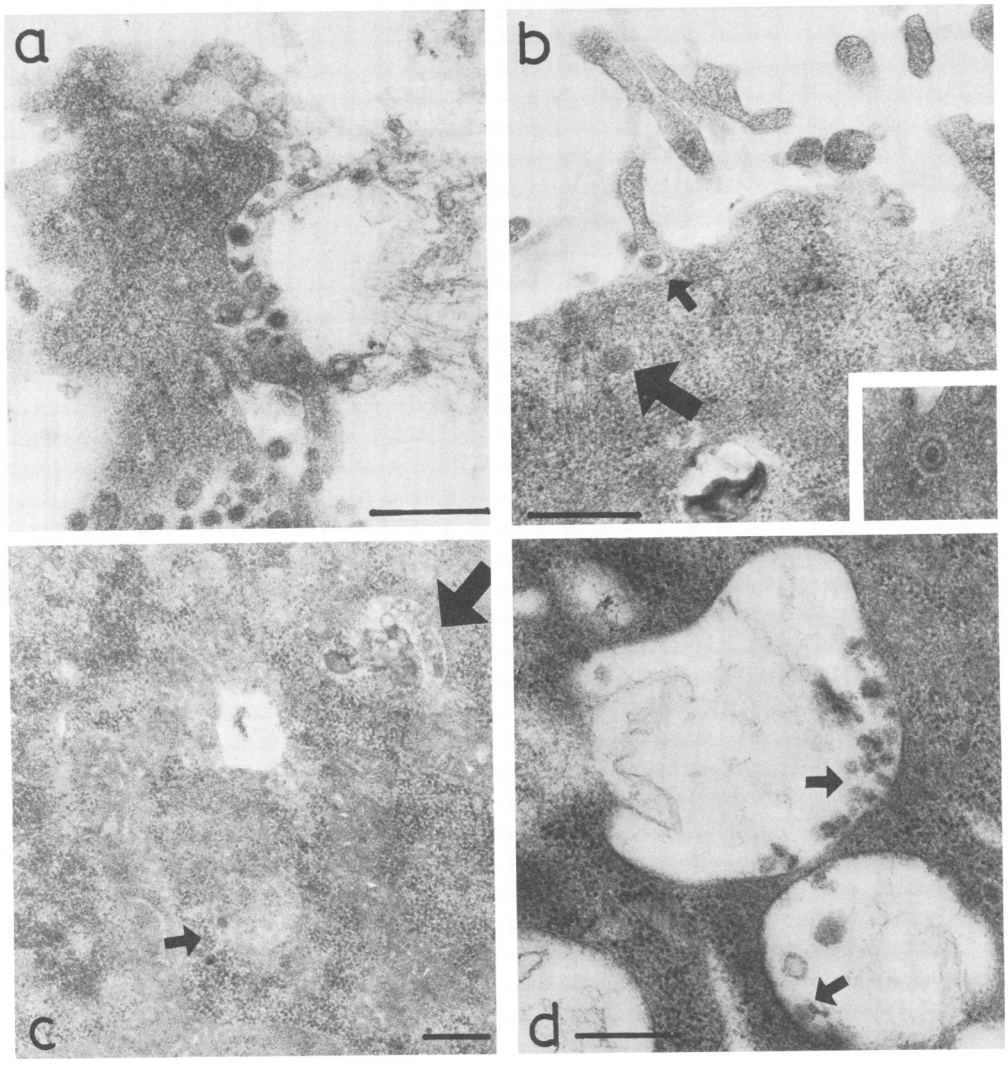

FIG. 4. Virus entry into MDCK cells and effect of chloroquine. Virus was allowed to adsorb to MDCK monolayers at neutral pH in the absence (a, b, c) or presence (d) of 0.1 mM chloroquine and was warmed to 37°C for 0 min (a), 5 min (b), 10 min (c), or 15 min (d). The specimen was fixed, sectioned, and viewed under an electron microscope as described in the text. Bar, 500 nm. After 5 min (b), virus particles in coated pits (small arrow) and coated vesicles (large arrow, also in the inset) are seen. After 10 min (c), virus particles in smooth-surfaced vacuoles (small arrow) and secondary lysosomes (large arrow) can be seen. In the presence of chloroquine (d), many virus particles remained inside vacuoles (small arrow) after 15 min.

the foot of microvilli, and internalized into coated vesicles (Fig. 4b). After 10 min, the virus was found in smooth-surfaced vacuoles and secondary lysosomes (Fig. 4c). After 15 min, regular-shaped virus particles were not recognizable in the vacuoles and secondary lysosomes (data not shown).

For quantitative assay of endocytosis, we utilized reduction and reoxidation of the head-group spin label Tempo-PC incorporated into the virus envelope. Figure 5a shows the time course of the ESR signal intensity from spin labels on the virus accessible to the externally added ferricyanide, when the inoculum was incubated at 37°C. The signal intensity therefore approximates the number of virus particles bound on the cell surface. The unrestored signal was due to the spin labels on the endocytosed virus particles, which were protected from the externally added ferricyanide. If some virus was released from the cell surface during incubation by the viral neuraminidase action and lost on

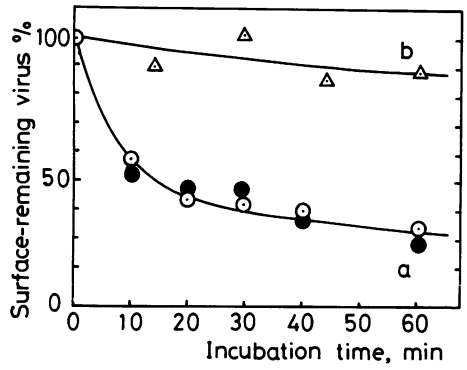

FIG. 5. Assay of virus particles remaining bound on the cell surface. Tempo-PC-labeled virus was pretreated with ascorbate. The virus (3,000 HAU/ml) was adsorbed to MDCK cell suspension (5×10^6 cells per ml) and incubated in MEM-HEPES at neutral pH for varying periods. Samples were withdrawn and centrifuged. ESR spectrum of the pellet was measured in the presence of ferricyanide. Percentage of the surface-remaining virus was calculated from the ratio of ESR peak height at time t to that at time 0. Curve a: In the absence (○) or presence (●) of 0.1 mM chloroquine. Curve b: A control experiment in which cells were pretreated with glutaraldehyde (2.5%) in PBS for 30 min at room temperature.

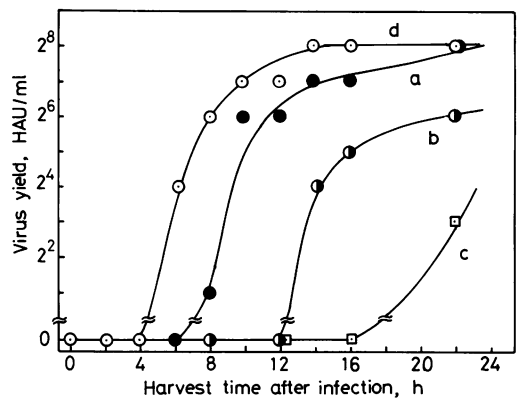

FIG. 6. Inhibition of virus replication by chloroquine. MDCK cell monolayers were pretreated with 0.1 mM chloroquine for 30 min at 37°C. Virus was added to the monolayer at a multiplicity of 10 PFU/cell and kept for 1 h on ice. The medium was replaced with MEM-HEPES (pH 7.8) containing chloroquine, and monolayers were incubated at 37°C for 0 h (a), 2 h (b), or 4 h (c). The medium was then removed and fresh MEM not containing chloroquine was added. Virus was harvested at the indicated time after inoculation. Curve d is a control in which all procedures were carried out in the absence of drug.

washing, a correction must be made for the estimation of endocytosed virus. However, the amount of released virus was found to be very small for the WSN-MDCK cell system. Plaque formation assay of the supernatant after centrifugation of the incubation mixture showed that about 10% of the initially adsorbed virus was released after 1 h of incubation at 37°C. Figure 5b shows the time course of the ESR signal intensity for the same incubation system used with glutaraldehyde-treated cells. It is probable that the endocytosis is largely inhibited in this system and the small signal decrease (about 10% after 1 h) can be due to released virus. These results suggest that a half-time of endocytosis is about 7 min at 37°C and that about 50% of the initially bound virus is internalized after 1 h. Chloroquine did not affect the time course (Fig. 5a).

Temperature dependence of endocytosis was studied by measuring ESR signal intensity after 1 h of incubation at various temperatures. There was a characteristic temperature around 20°C, below which the endocytosis was very slow and above which it became increasingly faster with temperature. It was confirmed that endocytosis during our spin-label assay (a few minutes at 22 to 23°C) was negligibly small.

Effect of chloroquine on virus replication. A lysosomotropic weak base chloroquine inhibited virus replication in MDCK cells. Figure 6 shows the inhibition when the monolayer cells were exposed to the drug before and after the inoculation. The drug caused a delay in the virus appearance in the medium and a reduction of

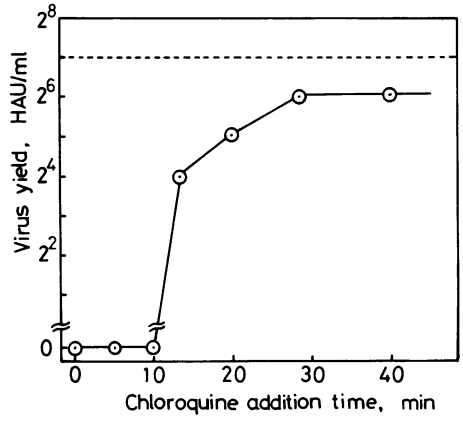

FIG. 7. Time dependence of chloroquine inhibition. MDCK monolayers were allowed to adsorb virus (10 PFU/cell) and were washed with PBS. Warmed MEM was added to the monolayer and, at the indicated time, new MEM containing 0.1 mM chloroquine at neutral pH was added. After 3 h of incubation at 37°C, the medium was replaced with fresh MEM not containing chloroquine, and monolayers were incubated for a further 6 h. The dotted line shows the level of virus yield in the absence of chloroquine.

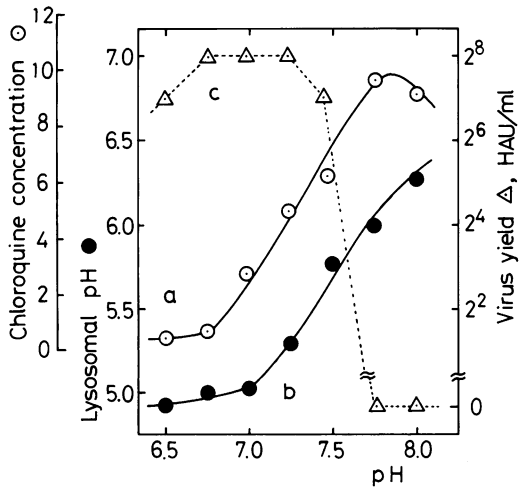

FIG. 8. pH dependence of chloroquine accumulation into cells (a), lysosomal pH (b), and inhibition of the virus replication by the drug (c). MDCK cell monolayers in 60-mm petri dishes were incubated for 20 min at 37°C in 4 ml of MEM-HEPES containing 0.1 mM chloroquine at various pH values. The intracellular chloroquine concentration was determined by fluorometry after solubilization of cells by deoxycholate as described in the text and expressed in units of 10^{-16} mol/cell. The intralysosomal pH was determined by fluorometry after ingestion of fluorescein isothiocyanate-labeled dextran as described in the text. For the assay of virus replication, virus was adsorbed to the cell monolayers at 10 PFU/cell at neutral pH and incubated for 4 h at 37°C in MEM-HEPES containing 0.1 mM chloroquine at various pH values. The medium was then replaced with new MEM not containing chloroquine, and monolayers were incubated for a further 6 h at 37°C at neutral pH.

virus yield. The delay became longer and the reduction was larger when chloroquine was present for longer time after inoculation. Cytopathic effect was small even after 4 h in the presence of chloroquine in the medium, as judged by cell rounding. Figure 7 shows that the drug acts on early event(s) of the virus replication in MDCK cells. Whereas the drug inhibited replication strongly when added at the time of inoculation or up to 10 min after inoculation, the inhibitory action was progressively weakened when the drug was added after 10 min. This mode of action of chloroquine is similar to those of amantadine derivatives reported previously (12, 15, 16, 33).

The intracellular chloroquine concentration and lysosomal pH of MDCK cells were measured after equilibration in chloroquine-containing media at various pH values (Fig. 8a and b). The chloroquine accumulation was optimum at pH 7.8; it decreased markedly when the medium pH was lowered beyond this point, and it became only slight at pH 6.5. Correspondingly, the lysosomal pH increased to a high value of 6.1 when equilibrated in the medium of pH 7.8, but decreased when the medium pH was lowered. The value returned to 4.9, the normal lysosome pH, when the medium pH was 6.5. The inhibition of virus replication by chloroquine diminished in relation to the decrease in intracellular chloroquine concentration and lysosomal pH (Fig. 8c). Virus reproduction was completely inhibited when the lysosomal pH was higher than 6.0 but was observed when the lysosomal pH was lower than 5.7. It is interesting that this pH dependence can be well correlated to that of envelope fusion (Fig. 3B). The lysosomal pH changes took place very rapidly. When cells were exposed to chloroquine-containing medium at pH 7.8, the lysosomal pH increased to 6.1 within 1 min. This is analogous to the case of macrophage (27). When the medium was changed for an acidic one (pH 5.0), the lysosomal pH dropped to 5.3 in 1 min at 37°C.

Electron micrographs also showed a marked effect of chloroquine. Many more virus particles were entrapped inside vacuoles and secondary lysosomes when cells were incubated in the presence of drug (Fig. 4d), in contrast to only a few particles observed in its absence (Fig. 4c).

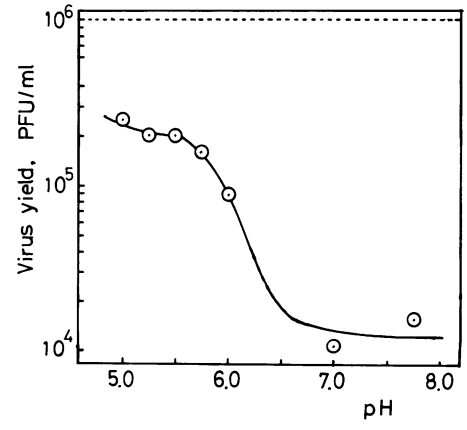

FIG. 9. Virus replication by brief exposure to acidic media. MDCK cell monolayers were pretreated with 0.1 mM chloroquine at pH 7.8. Virus was adsorbed to the monolayers at 4°C for 1 h at a multiplicity of 50 PFU/cell and exposed for 2 min at 37°C to BSS containing 0.1 mM chloroquine at various pH values. The cells were washed with MEM at pH 7.8 containing the drug and incubated for 3 h at 37°C. The medium was replaced with fresh MEM at neutral pH not containing chloroquine, and cells were incubated for a further 6 h at 37°C in the presence of trypsin. The dotted line shows the level for the virus yield when incubated only in the absence of drug at neutral pH.

TABLE 1. Effect of drugs on virus replication[a]

Drugs	Concn (mM)	Endocytosis (%)	Virus yield (PFU/ml) with drug treatment at times:	
			Before (30 min) and after (3 h) infection	1 h after infection
Chloroquine	0.1	50	1.5×10^4	1.8×10^6
NH_4Cl	10		1.8×10^4	1.9×10^6
Methylamine	20		2.9×10^4	2.4×10^6
Dansylcadaverine	0.05		6.2×10^4	2.6×10^6
Lidocaine	0.5		7.5×10^4	2.8×10^6
Neutral red	0.1		1.5×10^4	3.0×10^6
Cytochalasin B	0.02	40	2.4×10^6	5.5×10^6
Colchicine	0.1	50	4.3×10^6	4.5×10^6
Trifluoroperazine	0.025	45	4.5×10^4	1.0×10^5
W-7	0.05	45	3.0×10^4	4.5×10^4
Sulfatides from *M. tuberculosis*[b]	0.5 mg/ml		1.6×10^5 (16 h)	
Control		50		6.0×10^6

[a] MDCK cell monolayers were pretreated with various drugs at the indicated concentrations for 30 min at 37°C. Virus was added to the monolayers at a multiplicity of 10 PFU/cell, washed, and incubated for 3 h at 37°C in MEM containing the drug at neutral pH. The medium was replaced with fresh MEM not containing the drug and incubated for a further 6 h at 37°C in the presence of trypsin (5 μg/ml). In another series of experiments, the drug was added to the inoculum after 1 h of incubation at 37°C, and the inoculum was incubated for 3 h in the presence of drug. The medium was replaced with MEM not containing the drug, and the inoculum was incubated for a further 5 h at 37°C. Endocytosis was estimated from the ESR peak height of Tempo-PC-labeled virus as described in the text and expressed as a percentage of the initially bound virus after 1 h of incubation at 37°C. A 10% correction was made for the released virus.

[b] Cells were preincubated for 16 h at 37°C in MEM containing sulfatides.

Effect of various drugs on virus replication. The effect of drugs on virus reproduction is summarized in Table 1. Lysosomotropic weak bases inhibited the replication in a manner similar to that of chloroquine. Calmodulin inhibitors also showed marked inhibition. A characteristic difference from the lysosomotropic reagents is that these drugs were effective even when added 1 h after the inoculation. Cytoskeletal drugs did not show large inhibition. These drugs did not affect, or only slightly affected, the endocytosis. Sulfatides also showed a large inhibition of replication.

Virus replication by brief exposure to acid. When the inoculum was exposed to acidic medium, the virus envelope rapidly fused with the cell membrane, and viral RNA was transferred into the target cytoplasm. This may cause reproduction of the virus. This possibility was examined by briefly exposing the inoculum to acidic medium, then shifting to neutral medium and incubating at 37°C for 3 h, always in the presence of chloroquine. The medium was then replaced with MEM not containing the drug, and the inoculum was incubated for a further 6 h at 37°C. The results showed virus replication when the multiplicity was raised to 50 PFU/cell (Fig. 9). At 10 PFU/cell, as in the normal replication experiment at neutral pH, acid-induced virus replication (pH 5.0) was not observed. When the multiplicity was increased further to 100 PFU/cell, the virus yield was much smaller, probably because of cell lysis under this condition (data not shown). Acid exposure of cells by itself did not affect virus reproductivity. The pH dependence of virus replication was similar to that of envelope fusion, hemolysis, and cell fusion (Fig. 3), but appeared to shift to higher pH values.

DISCUSSION

The present study provides a strong support for the infectious cell entry mechanism of influenza virus, based on virus uptake by endocytosis, fusion of endocytosed vesicles with lysosomes, and finally fusion of the virus envelope with the surrounding vesicle membrane owing to low lysosomal pH (22, 24). The virus binding and uptake by endocytosis are shown in electron micrographs for the WSN virus strain-MDCK cell system, similarly to other strains of influenza virus and chick chorioallantoic membranes

(2, 4, 29). Endocytosis of the virus was confirmed by the spin-label assay using Tempo-PC, which gives about 7 min for the half-time. On the other hand, evidence for envelope fusion at neutral pH was not obtained either by electron microscopy or by the spin-label (PC*) assay. In the spin-label assay, we measured 10^9 virus particles and should observe a large (seven- to eightfold) increase in the ESR peak height if these viruses were fused with cell membranes (see Materials and Methods). Nevertheless, the result gave only negligible transfer of phospholipids. Therefore, envelope fusion with plasma membrane (fusion from outside) is unlikely to be an important infectious entry mechanism unless the local pH drops by more than 1 pH unit.

Fusion of lysosomes with phagosomes and endocytosed vesicles is known to occur in many cells. For example, 10 to 50% of the internalized phagosomes were reported to fuse with lysosomes in 20 min at 37°C in mouse macrophages (13). Rapid fusion of the endocytosed vesicles with lysosomes was shown by electron microscopy in the present system. Inhibition of the virus replication by sulfatides also supports the involvement of this fusion step; Goren et al. observed inhibition of phagosome-lysosome fusion when sulfatide-treated yeasts were ingested in macrophages (8).

The central event in the proposed infection sequence is fusion of the virus envelope with the endocytosed vesicle membrane, which causes transfer of the viral RNA into cytoplasm. The present study clearly shows that this fusion event is possible since the lysosomal pH of MDCK cells is 4.9 and at this pH envelope fusion takes place very rapidly (Fig. 3B). Chloroquine inhibits virus replication by blocking this fusion event; it rapidly raises the lysosomal pH to 6.1 (Fig. 8b), and the envelope fusion at pH 6.1 is negligible (Fig. 3B). The chloroquine inhibition was weakened in acidic media (Fig. 8c) because the lysosomal pH was lowered to 4.9 under these conditions (Fig. 8b). The pH dependence of the virus replication inhibition by chloroquine can be well explained by pH dependence of envelope fusion in the secondary lysosomes.

Chloroquine acts on some early event(s) in the infection sequence (within 10 min) but does not affect virus binding, endocytosis, or envelope fusion at pH 5.0. When the drug was added sometime (for example, 20 min) after inoculation, endocytosis was over 90% completed (Fig. 5a) and virus replication was not much inhibited (Fig. 7). These results support chloroquine action on lysosomes and also suggest that the drug does not affect later steps of virus reproduction for the present system. When virus-cell associates were incubated in the presence of chloroquine, many virus particles remained inside vacuoles and secondary lysosomes. This is consistent with the failure of envelope fusion in the lysosomes due to the increased pH, although hydrolytic digestion of virus particles may have also been weakened as a result of the increased pH.

Virus replication after brief exposure of the inoculum to acidic medium (Fig. 9) was probably due to the transfer of viral genome into cytoplasm by direct fusion with the cell surface membrane (fusion from outside). However, although chloroquine was always present in the medium, infection via fusion from inside is also possible during the 2-min exposure to acid since the lysosomal pH rapidly dropped to 5.3 during this exposure. Therefore, if some fractions of virus are endocytosed and sent to lysosomes, then envelope fusion should occur in the secondary lysosomes. A higher shift of the pH dependence for virus replication than those for envelope fusion and cell fusion may be partly responsible for the involvement of this infection route from inside.

The above infection mechanism for influenza virus is essentially the same as that proposed and developed for Semliki Forest virus (9, 35). Trapping of virus into coated pits, internalization by endocytosis into coated vesicles, and sequestering into intracellular vacuoles and lysosomes have been observed. Activation of envelope fusion and cell fusion in acidic media, inhibition of the virus replication by chloroquine, and virus replication induced by brief low-pH treatment of the inoculum have also been reported. There can be still other viruses which enter the target cells by endocytosis and uncoat the envelope by fusion in the secondary lysosomes. Acid-activation of hemolysis, envelope fusion, and cell fusion, as well as inhibition of virus replication by lysosomotropic weak bases, may indicate the presence of such an infection route.

We have proposed that the acid-activation of viral activity can be attributed to structural change(s) caused by protonation of the acidic residues in the amino-terminus arm of hemagglutinin HA_2 (22). Our preliminary data show that the characteristic ordered structure of hemagglutinin spikes disappeared and the envelope was disrupted after incubation for 5 min at pH 5.2 at 37°C. This is in parallel to rapid inactivation of hemolysis activity of influenza virus when incubated in acid at 37°C. The viral hemagglutinin proteins form a trimer in the envelope (37). The acid treatment may dissociate the trimer to expose the amino-terminus arm to interact with target membrane lipids or, in the absence of target, may cause them to entangle with each other irreversibly. Dissociation of

protein dimers has been induced by protonation of acidic residues for a water-soluble protein, lysozyme (34).

ACKNOWLEDGMENTS

We are greatly indebted to Sakuji Toyama and Akira Ishihama of Institute for Virus Research, Kyoto University, for their very helpful discussions, to Shoji Ohkuma of Teikyo University for his help, to H. Hidaka of Mie University for W-7, and to M. Kino for supplying us with *M. tuberculosis*.

ADDENDUM IN PROOF

Matlin et al. (K. S. Matlin, H. Reggio, A. Helenius, and K. Simons, J. Cell Biol. **91**:601–613, 1981) have recently obtained the same conclusion as the present study on the infectious cell entry mechanism of the influenza virus fowl plague strain.

LITERATURE CITED

1. **Bablanian, R. H., H. J. Eggers, and I. Tamm.** 1965. Studies on the mechanism of poliovirus-induced cell damage. I. The relation between poliovirus-induced metabolic and morphological alterations in cultured cells. Virology **26**:100–113.
2. **Dales, S., and P. W. Choppin.** 1962. Attachment and penetration of influenza virus. Virology **18**:489–493.
3. **Donald, H. B., and A. Isaacs.** 1954. Counts of influenza virus particles. J. Gen. Microbiol. **10**:457–464.
4. **Dourmashkin, R. R., and D. A. J. Tyrrell.** 1974. Electron microscopic observations on the entry of influenza virus into susceptible cells. J. Gen. Virol. **24**:129–141.
5. **Fraker, P. J., and J. C. Speck, Jr.** 1978. Protein and cell membrane iodination with a sparingly soluble chloroamide, 1, 3, 4, 6-tetrachloro-3a, 6a-diphenylglycoluril. Biochem. Biophys. Res. Commun. **80**:849–857.
6. **Gaush, C. R., and T. F. Smith.** 1968. Replication and plaque assay of influenza virus in an established line of canine kidney cells. Appl. Microbiol. **16**:588–594.
7. **Goren, M. B.** 1970. Sulfolipid I of Mycobacterium tuberculosis, strain $H_{37}R_v$. I. Purification and properties. Biochim. Biophys. Acta **120**:116–126.
8. **Goren, M. B., P. D. Hart, M. R. Young, and J. A. Armstrong.** 1976. Prevention of phagosome-lysosome fusion in cultured macrophages by sulfatides of Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. U.S.A. **73**:2510–2514.
9. **Helenius, A., J. Kartenbeck, K. Simons, and E. Fries.** 1980. On the entry of Semliki Forest virus into BHK-21 cells. J. Cell Biol. **84**:404–420.
10. **Huang, R. T. C., R. Rott, and H.-D. Klenk.** 1981. Influenza viruses cause hemolysis and fusion of cells. Virology **110**:243–247.
11. **Huang, R. T. C., K. Wahn, H.-D. Klenk, and R. Rott.** 1980. Fusion between cell membrane and liposomes containing the glycoproteins of influenza virus. Virology **104**:294–302.
12. **Kato, N., and H. J. Eggers.** 1969. Inhibition of uncoating of fowl plague virus by 1-adamantanamine hydrochloride. Virology **37**:632–641.
13. **Kielian, M. C., and Z. A. Cohn.** 1980. Phagosome-lysosome fusion. Characterization of intracellular membrane fusion in mouse macrophages. J. Cell Biol. **85**:754–765.
14. **Klenk, H.-D., R. Rott, M. Orlich, and J. Blödorn.** 1975. Activation of influenza A viruses by trypsin treatment. Virology **68**:426–439.
15. **Koff, W. C., and V. Knight.** 1979. Inhibition of influenza virus uncoating by rimantadine hydrochloride. J. Virol. **31**:261–263.
16. **Koff, W. C., and V. Knight.** 1979. Effect of rimantadine on influenza virus replication. Proc. Soc. Exp. Biol. Med. **160**:246–252.
17. **Kornberg, R. D., and H. M. McConnell.** 1971. Inside-outside transition of phospholipids in vesicle membranes. Biochemistry **10**:1111–1120.
18. **Lazarowitz, S. G., and P. W. Choppin.** 1975. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology **68**:440–454.
19. **Lenard, J., and D. K. Miller.** 1981. pH-dependent hemolysis by influenza virus, Semliki Forest virus, and Sendai virus. Virology **110**:479–482.
20. **Maeda, T., A. Asano, K. Ohki, Y. Okada, and S. Ohnishi.** 1975. A spin label study of fusion of red blood cells induced by hemagglutinating virus of Japan. Biochemistry **14**:3736–3741.
21. **Maeda, T., A. Asano, Y. Okada, and S. Ohnishi.** 1977. Transmembrane phospholipid motions induced by F glycoprotein in hemagglutinating virus of Japan. J. Virol. **21**:232–241.
22. **Maeda, T., K. Kawasaki, and S. Ohnishi.** 1981. Interaction of influenza virus hemagglutinin with target membrane lipids is a key step in virus-induced hemolysis and fusion at pH 5.2. Proc. Natl. Acad. Sci. U.S.A. **78**:4133–4137.
23. **Maeda, T., K. Kuroda, S. Toyama, and S. Ohnishi.** 1981. Interaction of hemagglutinating virus of Japan with erythrocytes as studied by release of a spin probe from virus. Biochemistry **20**:5340–5345.
24. **Maeda, T., and S. Ohnishi.** 1980. Activation of influenza virus by acidic media causes hemolysis and fusion of erythrocytes. FEBS Lett. **122**:283–287.
25. **Maeda, T., and S. Ohnishi.** 1982. Interaction of enveloped virus with cell membranes. A spin label study. *In* R. Sato (ed.), Structure, dynamics, and morphogenesis of biomembranes, in press. Japan Science Society Press, Tokyo.
26. **Morgan, C., and H. M. Rose.** 1968. Structure and development of viruses as observed in the electron microscope. VIII. Entry of influenza virus. J. Virol. **2**:925–936.
27. **Ohkuma, S., and B. Poole.** 1978. Fluorescence probe measurement of the intralysosomal pH in living cells and the perturbation of pH by various agents. Proc. Natl. Acad. Sci. U.S.A. **75**:3327–3331.
28. **Okada, Y., I. Koseki, J. Kim, Y. Maeda, T. Hashimoto, K. Kanno, and Y. Matsui.** 1975. Modification of cells with viral envelopes during fusion of cells with HVJ (Sendai virus). I. Interaction between cell membrane and virus in the early stage. Exp. Cell. Res. **93**:368–378.
29. **Patterson, S., J. S. Oxford, and R. R. Dourmashkin.** 1979. Studies on the mechanism of influenza virus entry into cells. J. Gen. Virol. **43**:223–229.
30. **Poste, G., and G. L. Nicolson (ed.).** 1977. Cell surface reviews, vol. 2: Virus infection and the cell surface. North-Holland Publishing Co., Amsterdam.
31. **Quintart, J., M.-A. Leroy-Houyet, A. Trouet, and P. Baudhuin.** 1979. Endocytosis and chloroquine accumulation during the cell cycle of hepatoma cells in culture. J. Cell Biol. **82**:644–653.
32. **Salk, J. E.** 1944. A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. J. Immunol. **49**:87–98.
33. **Skehel, J. J., A. J. Hay, and J. A. Armstrong.** 1979. On the mechanism of inhibition of influenza virus replication by adamantadine hydrochloride. J. Gen. Virol. **38**:97–110.
34. **Sophianopoulos, A. J., and K. E. van Holde.** 1964. Physical studies of muramidase (lysozyme). II. pH-dependent dimerization. J. Biol. Chem. **239**:2516–2524.
35. **White, J., J. Kartenbeck, and A. Helenius.** 1980. Fusion of Semliki Forest virus with the plasma membrane can be induced by low pH. J. Cell Biol. **87**:264–272.
36. **White, J., K. Matlin, and A. Helenius.** 1981. Cell fusion by Semliki Forest, influenza, and vesicular stomatitis viruses. J. Cell Biol. **89**:674–679.
37. **Wilson, L. A., J. J. Skehel, and D. C. Wiley.** 1981. Structure of the haemagglutinin membrane glycoprotein. Nature (London) **289**:366–373.